Detection of anti-ribosome antibodies: A long story of lights and shadows

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ABSTRACT

Circulating autoantibodies against ribosomal proteins characterise a subset of patients with systemic lupus erythematosus. Following the identification of three phosphorylated proteins as the main ribosomal autoantigens recognised by these autoantibodies, several studies have been carried out in the last decade to set up a reliable and sensitive method of detecting anti-ribosome autoantibodies and disclosing their possible clinical relevance in the diagnosis and monitoring of symptoms and signs of the disease. Although a number of clinical associations have been proposed, contrasting results have emerged from these investigations. This review analyses the methodological problems linked with the various techniques used to detect anti-ribosome antibodies and provides a critical update of the clinical associations described in lupus patients to date.

Introduction

Systemic lupus erythematosus (SLE) is characterised by the presence of a wide array of serum autoantibodies. While most of these are directed against nuclear antigens, a subset is directed to ribosomal RNAs and proteins, mainly located in the large 60S subunit of mammalian ribosomes (reviewed in Ref. 1). Within these ribosomal constituents, SLE sera mainly recognize three phosphoproteins (Proteins) with molecular weights of 38 (P0), 19 (P1), and 17 (P2) kD, respectively (1). Other antigenic specificities, including L12, a 20 kD 60S subunit protein (2-4), S10, a 20 kD 40S subunit protein (4, 5), L5, a 35 kD protein constituent of the L5/5S ribonucleoprotein complex (6), and 28S ribosomal RNA (7), are less frequently recognized.

Autoantibodies directed to the P proteins are the best characterized anti-ribosomal antibodies (8), while those against other ribosomal constituents have been less thoroughly studied. The clinical relevance of anti-P antibodies derives from their disease restriction to SLE, their associations with certain clinical and serological features in SLE patients, and their in vitro pathogenicity. Many studies targeting these features have been published in recent years, although contrasting data have often been reported. One reason for these discrepancies could be based on differences in the methodological approach employed to detect anti-ribosome antibodies. However, a number of other explanations such as differences in ethnic origin of the patient groups studied, type of study (retrospective or prospective), size of the patient cohort, inclusion criteria and clinical evaluation of disease activity and organ involvement, have all been suggested. This review will examine the methodological problems associated with the different approaches used to detect anti-ribosome antibodies and will focus on the main clinical and serological associations revealed by studies in the last few decades.

Early studies

The techniques established to detect anti-P antibodies have been conditioned by the various attempts made to identify the nature of the antigen within ribosomes. When the precise target of anti-P antibodies was not clear, techniques involving whole ribosomes as antigen or tissue substrates were the only choice. With this techniques, indirect evidence of the protein or ribonucleic nature of the antigen was obtained by treating the substrate with proteolytic or ribonuclease enzymes. It was not until the mid-1980s that the proteic nature of the antigen was clarified and new techniques were designed in order to both increase the performance of the tests and refine the epidemiological data.

Sturgill and Carpenter were the first to discover evidence of antibodies directed towards ribosomes in the sera of...
patients with SLE; in 1965 they found this type of specificity in 23% of lupus patients using partially purified ribosomal fractions (9). In reality another study just a few years earlier had examined the pathogenic potential of induced anti-ribosomal antibodies in animals immunized with ribosomes (10). These authors attempted to identify the nature of the antigen (protein or nucleic acid) by selective immunization with whole ribosomes, protein or RNA fractions (11). Their results suggested that the induced immune reaction was mainly directed towards nucleic acids, rather than against proteins.

A study published several years later confirmed the presence of anti-ribosomal antibodies in 12.9% of SLE patients (12). The authors used agar immunodiffusion with whole ribosomes as antigens. The use of enzymes to disrupt the ribonucleic acid or protein structures partly abolished the antigen reactivity, suggesting that both components play a role.

A study conducted in 1970 searched for anti-ribosomal antibodies in 50 SLE sera using ribosome-coated latex particles and double diffusion in agar. The results, showing 18% positive sera by latex and 8% positive by immunodiffusion, demonstrated that even when using the same type of antigen, the sensitivity could be affected by the technique used.

The first study of anti-ribosomal antibodies identified by immunofluorescence was published in 1974 (14). The authors compared various tissues in order to discover one which would allow unequivocal identification of the anti-ribosomal specificity without confusion with other anti-ribonucleic acid seeing that the antigen was non-organ and non-species specific. Soon afterwards evidence emerged that the antigen was highly conserved. Another study published in the same year provided more direct evidence for the target of anti-ribosomal antibodies: they bound ribosomes as shown by electronic microscopy (15). In addition, digestion studies led to the conclusion that the main antigens recognized by anti-ribosomal antibodies were composed of proteins.

A liquid phase radioimmunoassay with whole triturated ribosomes was set up in 1977 (16). It was the first attempt to improve the sensitivity of the test also enabling it to quantify the immune response. However, an unusually high percentage of SLE patients was found to be positive with this test (63-77%) and many rheumatoid arthritis and chronic hepatitis patients were also shown to be positive: apparently, the gain in sensitivity decreased the good specificity obtained using other techniques. In fact, a subsequent epidemiological study enrolling patients with different connective tissue diseases confirmed the high SLE specificity of anti-ribosomal antibodies detected by immunodiffusion, since they were only rarely found in patients with other connective tissue diseases (17). In the same year, a solid phase radioimmunoassay for anti-ribosomal antibodies was also developed (18). The original procedure was set up using whole ribosomes and radiolabelled anti-IgG from a few anti-ribosomal sera, but the author suggested that the assay could be improved by using purified proteins. This test, in spite of its limitations, was the basis for subsequent development of immunoonassays. Because the precise target of anti-ribosomal antibodies was still unclear, another study employing the aforementioned liquid phase radioimmunoassay was carried out in SLE sera (19). The results pointed to a reactivity of anti-ribosomal antibodies against either protein or RNA antigens, although the latter were mainly present in patients with inactive disease, whereas ribonucleoprotein particles were predominantly recognized by sera from patients with active disease. Anyway, only one-third of the antibodies detected by radioimmunoassay were able to react by immunodiffusion in agar, while no anti-RNA antibody showed this property. This confirmed that the sensitivity of this method was higher than that of immunodiffusion, although the lack of sufficient evidence for the superiority of one technique over the others to detect these autoantibodies, frequently forced researchers to employ more than one method in that years.

Another study was performed in 1980 in order to refine the binding ability of anti-ribosomal antibodies on different tissues by immunofluorescence (20). Although the authors employed a single patient serum, they confirmed that, although anti-ribosomal antibodies reacted with different tissues, some of these were better than others for distinguishing anti-ribosome from antimitochondria autoantibodies. Two years later, 322 patients with different connective tissue diseases, including 171 subjects with SLE, were tested for anti-ribosomal antibodies in immunofluorescence: 13 of them (11 from SLE sera) were found to be positive and in most cases the sera reacted in immunodiffusion with whole ribosomes (21). Another study (22) compared the antigens bound by anti-ribosomal antibodies in immunofluorescence performed on different tissues and cell lines and immunodiffusion on purified ribosomes. Serum adsorptions with whole ribosomes or with RNase- or trypsin-treated ribosomes were performed and the residual binding ability was verified. The results suggested the different nature of the ribosomal antigen since only whole ribosome adsorption was able to abolish the anti-ribosomal binding activity. In addition, a clinical evaluation in the same study pointed to a preferential association of anti-ribosomal antibodies with active disease and, in particular, with renal involvement. Until then, all the attempts at biochemical characterization of ribosomal antigens had been indirect, using protease or ribonuclease, adsorbing sera with ribosomes or RNAbefore testing, etc.

Identification of Proteins
The first unequivocal evidence that antibodies directed against ribosomes reacted with antigenic determinants located on proteins occurred in 1982 (23). Ribosomal proteins were electrophoretically separated by SDS-PAGE, transferred onto a nitrocellulose
membrane and an immunoblot with sera from patients with various connective tissue diseases was performed. Interestingly, two of the three authors of these studies were pioneers of the use of SDS-PAGE technique and nitrocellulose sheet as support for immunological binding. With this method, 23% of sera from SLE resulted positive. Moreover, solid-phase enzyme-linked immunosassays on microtiter plates with extracted proteins or whole ribosomes were used to set up a confirmatory enzyme-linked immunosassay test, which proved to be a sensitive screening test, overcoming the problems linked to radioactivity.

Subsequently, the same group of authors immunized mice with whole ribosome in order to identify the protein target of autoantibodies (24). They obtained a monoclonal antibody able to cross-react to P1 and P2 proteins and also reacted with a third protein of 38 KDa that they named P0. The three proteins were collectively called “P proteins”, where P means “phosphorylated”. This important work first demonstrated the immunological cross-reactivity of the three ribosomal P proteins, i.e. it supplied the evidence that the three proteins shared a common antigenic determinant. They also showed the conservative nature of these proteins among eukaryotes, since the antibody reacted with the three P proteins obtained from a variety of sources.

The reactivity of anti-ribosomal antibodies spontaneously produced in humans was further characterised by two different groups in 1985 (25,26). The cross-reactivity was demonstrated directly, since eluted antibodies reacting to P0 in immunoblot, reacted not only with P0 but also with P1 and P2. The interspecies reactivity was also confirmed in one of the two investigations (26).

**Final step: the C-terminal sequence**

In 1986, a fundamental study was published (27). Since the abolition of anti-P reactivity had been observed with limited proteolytic digestion of ribosomes, it was supposed that the antigenic determinant resided at the end of the proteins. On the basis of the strong conservative nature of ribosomal proteins, Elkon et al. synthesized the 22-aminoacid C-terminal peptide of Artemia salina P2 protein, whose sequence was well-known, in order to verify whether it contained the cross-reactive antigen. The peptide was conjugated to carrier proteins and dot blot experiments confirmed the binding ability of anti-Ppositive SLE sera to this epitope. Moreover, the peptide was able to inhibit the binding capacity of autoantibodies to ribosomal proteins in blot. This was the first demonstration that such a sequence was involved in the anti-ribosomal reactivity.

The binding of anti-ribosomal antibodies to ribosomal 38, 19 and 17 kD proteins by immunoblot had been, until then, the best technique available for revealing anti-ribosomal activity. However, the identification of the cross-reactive epitope gave the opportunity to set up other techniques based on the detection of the synthesized aminoacid sequence, such as ELISAassays. These techniques were as reliable as immunoblot but offered several advantages: for example, they provide a quantitative evaluation of anti-P reactivity and are less time-consuming than ribosomal protein preparation. The identified epitope was used in different ways in many laboratories to set up reliable tests on solid phase, while both immunofluorescence and immunodiffusion were virtually abandoned.

Indeed, subsequent studies were performed mainly with immunoblot on SDS-PAGE separated ribosomal proteins and/or different immunosorbent tests, essentially ELISAs (3,28-30) based on the C-terminal aminoacid sequence shared by the three proteins. The ELISA techniques were generally set up in two different ways: in the first, the synthesized peptide was covalently linked to a carrier protein, serum albumin from various species or thyroglobulin, and then coated onto the solid phase (31-33). In the second one, the peptide was directly coated onto plates without any carrier. This approach also produced fruitful results, since the absorption by the solid phase did not prevent the antibody binding ability (34-36).

As above mentioned, both immunoblot and ELISA were initially used, but the demonstration in a number of studies that the two methods of anti-P antibodies detection gave overlapping results led to the employment of ELISA as a single technique in a number of subsequent investigations. In this context, it is important to underline that antibodies reacting in ELISA with the synthetic peptide and those reacting with all three P proteins in immunoblotting may be different. Antibodies reacting in immunoblot with only one of the three proteins (namely, against the largest P0 protein), are described, but they should not be considered anti-P autoantibodies, since they probably react towards an epitope different from the C-terminal sequence shared by the three Pproteins. In addition, the fact that some SLE sera show anti-P reactivity by ELISA but not immunoblot may be easily explained by the higher sensitivity of the former with respect to the latter method.

The C-terminal 22-aminoacid peptide sequence was reproduced by other authors in different investigations on anti-P antibodies, but a detailed study of epitope mapping performed on this sequence soon allowed restriction of the length of the epitope recognized by anti-Pantibodies to the last 11 C-terminal aminoacids (37). Moreover, it was shown that the use of the human P2 sequence, slightly different from that originally used (containing two conservative aminoacid replacements) did not seem to offer any advantage over the binding ability of the original synthesized sequence. Therefore, most authors continued to use the “classic” 22-aminoacid peptide as antigen, sometimes employing immunoblot as a confirmatory technique, despite the fact that epitope mapping clearly showed that the antigenic determinant is confined in a shorter sequence. An exception was made by Spezialetti et al. who used a 17 aminoacid peptide linked to BSAs carrier protein (38).

On the basis of the previously mentioned epitope mapping study (37) and in order to improve the methods of anti-P antibodies detection, one of us (L.C.) decided to synthesize a shorter sequence.
peptide (13 aminoacides) as a multiple antigen peptide (MAP), i.e. a core of Lysine from which four copies of the same 13-mer aminoacid peptide branch out (39). An ELISA employing this MAP was set up and its performance was compared to immunoblotting (the gold standard), and to simple peptide ELISA. The comparison confirmed the optimal binding activity of the shorter sequence and the good agreement with results obtained in immunoblot. Moreover, the comparison between MAP and single peptide ELISA pointed out the higher sensitivity of the former, presumably due to the better ability of the branched peptide, containing chains not involved in polymer binding, to be bound by antibodies. The choice to use a shorter peptide was fruitful for us and two recent study, performing a new fine epitope mapping of the C-terminus of P proteins, confirmed that the very last aminoacids seem to be the repository of antigenic reactivity, with a critical importance of the last five residues (40, 41).

Sporadically, antigens different from synthetic peptides, such as a recombinant P0 fusion protein coated on a solid phase in ELISA(42), have been used in the last few years. However, this recombinant antigen does not seem to offer advantages with respect to peptides, while it may also detect antibodies directed to epitopes located on P0 protein different from those shared with P1 and P2 proteins, as subsequently stated by the same authors (43).

A study comparing various assays for the detection of anti-P antibodies was published few years ago (44). ELISAs with 22-aminoacid MAP, affinity purified P proteins from bovine or human tissues, as coating antigens for ELISA, and immunoblot on SDS-PAGE separated P proteins of bovine or human origin were compared for their binding ability to known anti-P positive sera. The comparison confirmed the good performance of all techniques: the specificity was excellent and some sera, recognized as positive for anti-ribosomal antibodies by immunoblotting but negative by MAP ELISA, were actually directed to epitopes different from the C-terminal shared sequence. In fact, it has been shown that autoantibodies directed against P protein epitopes different from those located on the C-terminus may develop in SLE patients (45). Another recent comparison between immunoblotting and ELISAs with either C-22 single peptide and C-13 MAP resulted in good agreement and excellent specificity, although the sensitivity of immunoblotting and 13-aminoacid MAP-ELISA for weakly positive sera was better than that obtained with single peptide ELISA(46).

**Epidemiological evaluations**

Taken together, the studies carried out until now suggest that anti-Parabodies represent a hallmark of SLE, since they have been described only sporadically in connective tissue diseases other than SLE or SLE overlapping and transitional disorders (47,48). As shown in Table I, the frequency of anti-P antibodies, described in the most important papers published in the last years, ranges from 10 to 42% in SLE patients, although the number of patients enrolled in each study is highly variable. Three main features, race, age at disease onset and disease activity, appear to significantly favour the discrepant anti-P frequency in SLE, although the method of serological detection may be also important. Indeed, anti-P antibodies, are more frequently found in Asiatic patients than in Caucasian (in particular European), SLE populations and this appears to agree with the observation that their production is influenced by certain MHC class II alleles (49, 50, 51). Moreover, anti-P antibodies appear to be more frequent in SLE subjects with juvenile onset of the disease (52, 53, 54). Finally, an association of these autoantibodies with active SLE has been found in almost all studies that looked for it (see Table I for details). These findings, taken together, may suggest that anti-P response characterizes a subset of SLE subjects, in which genetic factors can favour early onset of a more severe disease.

**Serological associations and cross-reactivity**

Another interesting point regarding anti-P antibodies emerged from several studies: in general, anti-P antibodies are more frequently represented in sera where other SLE-specific autoantibodies are simultaneously present, namely anti-Sm and anti-dsDNA antibodies (Table I). This finding may be more than a simple coincidence. The hypothesis of a cross-reactivity between ribosomal P proteins and Sm antigen was proposed quite early by two groups (60, 61). A possible relationship between anti-P and anti-dsDNA antibodies has been postulated recently on the basis of the observation of high incidence of anti-P antibodies in SLE patients with renal involvement (57, 62), where the association with high serum levels of anti-dsDNA is well known. In fact, anti-dsDNA antibodies cross-reacting with ribosomal P proteins expressed on the surface of glomerular mesangial cells had been previously described (63). Recently, clear evidence of a population of anti-P antibodies able to react with specific Sm proteins or DNA has also been provided (64, 65). These findings are particularly intriguing due to the very high specificity of these three autoantibodies for SLE.

Association of anti-P with other autoantibodies has been sporadically reported (see Table I). In some cases these observations produced a significant correlation; in other cases only a trend was observed. However, it is a fact that the presence of anti-P antibodies with a limited number of other specificities appear more frequently. In this context, the association with anticardiolipin (aCL). IgG is of particular interest, since it has been confirmed in the majority of studies in which it has been correctly evaluated (31, 46, 54, 66). In contrast to the reported findings for anti-Sm and anti-dsDNA, the possibility of cross-reactivity between anti-P antibodies and aCL is still a matter of debate (67). However, it is intriguing that anti-P antibodies, although associated with aCL. IgG, are not usually associated with overt antiphospholipid syndrome (46, 54, 66). Moreover, although aCL are closely associated with central nervous system (CNS) manifestations essentially related to organic
Similarly, cerebral vasculopathy, anti-P appear to be more related to functional abnormalities of CNS. In addition, some kind of antiphospholipid antibodies specifically bind surface antigens on endothelial cells (68) as anti-P antibodies do (see below). Taken together, these observations suggest that aCL and anti-P may recognize epitopes shared by ribosomal protein antigens and phospholipid-binding proteins located within the CNS; however, further studies are needed to understand the pathogenetic and clinical relevance of these observations.

Another important issue, emerged from several studies, is that anti-P antibodies appear to be able to bind proteins located at the surface of various cell types. A first investigation showed that affinity purified anti-P antibodies may react with surface proteins on neuroblastoma and hepatoma cell lines (69). Other authors demonstrated that purified anti-P antibodies may bind other cell lines as glial cell lines or cells derived from lung cancer (70), or transformed T cell lines (70, 71).

More significantly, binding of anti-P antibodies to the surface of normal cell types such as endothelial cells (72), or T lymphocytes (71,73) has been shown. The target of anti-P antibodies may be a cell surface P0 protein (69, 70, 74) that, in some cases, might drive autoantibody penetration into the cells (70, 74).

Clinical associations
As widely described, the first recognition of anti-ribosomal antibodies in SLE dates from about 40 years ago, but the strongest association of these auto-antibodies with clinical manifestations of the disease was proposed in 1987, when high titers of circulating anti-P antibodies were described in patients.
with lupus psychosis (30). Since then, several studies have been published on this topic. However, clinical significance of anti-P antibodies in SLE subjects is still a matter of debate, since conflicting results on the relationship between anti-P antibodies and neuropsychiatric or other clinical manifestations in SLE have been reported until now (8,76,77). The results of the main papers on the subject published in recent years are summarized in Table II. The majority of these studies investigated the possible association of anti-P reactivity with SLE neuropsychiatric manifestations, undoubtedly the topic that most often captured the attention of investigators in recent years. The conflicting findings reported in the published studies investigating this association may have several explanations, including differences in the methodological approaches adopted for detecting antibodies (Table I). However, one of the main problems was the correct recognition of neuropsychiatric syndromes. Indeed, the majority of the studies concerning this entity suffered from a number of methodological limitations which may account for the considerable differences in prevalence of CNS involvement in SLE reported in the literature (78). The first point is that the definition of neuropsychiatric lupus includes a variety of manifestations, such as cerebrovascular accidents, seizure disorders, cranial and peripheral neuropathies, psychosis, mood and anxiety disorders and others, that are extremely heterogenous and that, at least in part, may not share a common pathogenetic pathway (79-82). Another important point is that neurological syndrome can be quite easily recognized and defined in SLE patients with the help of sophisticated imaging techniques, while precise classification of psychiatric syndromes, and clear delineation of the variables causally connected to psychiatric symptoms in these patients, often remains elusive (83-87). As shown in Table II, where the reported data on psychosis, other psychiatric manifestations such as mood as well as anxiety disorders, and neurological manifestations are summarized separately, the prevalent association of anti-P antibodies is with psychiatric symptoms, whereas the hypothesis of an association with neurologic lupus manifestations appears to be less supported. However, it is to note that, among the different psychiatric manifestations, psychosis appears to be that mainly associated with anti-P antibodies (and their serum titers), since the negative results reported in Table II essentially derives from studies with very few psychotic patients. Therefore, remaining doubts on anti-P antibodies and psychosis association may only be ruled out with the analysis of a wide cohort of patients suffering from this rather rare SLE manifestation, also in order to establish the possible clinical value of anti-P determination in monitoring treatment effect, as recently suggested (88).

Table II. Summary of the clinical associations found in SLE patients with anti-P antibodies (n.d.: not determinable).

<table>
<thead>
<tr>
<th>1st Author (Ref.)</th>
<th>Year</th>
<th>Association with psychosis</th>
<th>Association with other psychiatric manifestations</th>
<th>Association with neurologic manifestations</th>
<th>Association with other clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheneebaum (31)</td>
<td>1991</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sato (3)</td>
<td>1991</td>
<td>No</td>
<td>No</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Van Dam (35)</td>
<td>1991</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Discoid rash, photosensitivity, oral ulcers</td>
</tr>
<tr>
<td>Nojima (29)</td>
<td>1992</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Teh (32)</td>
<td>1992</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>n.d.</td>
</tr>
<tr>
<td>Teh (55)</td>
<td>1993</td>
<td>n.d.</td>
<td>Yes</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Yoshio (42)</td>
<td>1995</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Press (52)</td>
<td>1996</td>
<td>Yes</td>
<td>n.d.</td>
<td>n.d.</td>
<td>No renal involvement association</td>
</tr>
<tr>
<td>Isshi (33)</td>
<td>1996</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>n.d.</td>
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<tr>
<td>Arnett (49)</td>
<td>1996</td>
<td>Yes</td>
<td>Yes</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>Chindalore (57)</td>
<td>1998</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Renal involvement but not other</td>
</tr>
<tr>
<td>Reichlin (53)</td>
<td>1999</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Renal involvement (anti-P+ and anti-DNA+)</td>
</tr>
<tr>
<td>Tzioufas (36)</td>
<td>2000</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Malar and discoid rash, photosensitivity</td>
</tr>
<tr>
<td>Massardo (58)</td>
<td>2002</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Yalaoui (59)</td>
<td>2002</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Gerli (54)</td>
<td>2002</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Malar and discoid rash, photosensitivity</td>
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</table>
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- Autoantibodies directed against the ribosomal P proteins are not only directed against a common epitope of the P0, P1 and P2 proteins. J Autoimmunity 1999; 13: 103-10.